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QTL Analysis of Rice Grain Size Using Segregating Populations Derived from the Large Grain Line

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Abstract: Grain size affects the yield and quality of rice. The large grain line (LGL), showing a large grain size and *japonica*-like genome, was selected in the breeding field. The 94 F_2 plants derived from a cross between LGL and Hanareum (a high-yielding tongil-type variety) were used for the quantitative trait loci (QTL) analysis of grain length (GL), grain width (GW), and grain thickness (GT). A linkage map of the F_2 population, covering 1312 cM for all 12 chromosomes, was constructed using 123 Fluidigm SNP markers. A total of nine QTLs for the three traits were detected on chromosomes two, three, four, six, and seven. Two QTLs for GL on chromosomes two and six explained 17.3% and 16.2% of the phenotypic variation, respectively. Two QTLs were identified for GW on chromosomes two and three, and explained 24.3% and 23.5% of the phenotypic variation, respectively. The five QTLs for GT detected on chromosomes two, three, five, six and seven, explained 13.2%, 14.5%, 16.6%, 10.9%, and 10.2% of the phenotypic variation, respectively. A novel QTL for GT, *qGT2*, was validated on the same region of chromosome two in the selected F_3 population. The QTLs identified in this study, and LGL, could be applied to the development of large-grain rice varieties.

Keywords: rice; grain size; QTL; large grain

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1. Introduction

Rice is one of the most important grain crops in the world and is also the main source of calories for half of the world's population. Given the rapid growth of the global population, the world's population is predicted to reach nine billion by the middle of this century. The grain yield of rice should be increased by 70–100%, relative to the current levels, to feed the increasing global population [1,2]. The grain yield of rice is mainly affected by three components: the number of grains per panicle, the number of panicles per plant, and grain weight. Grain weight is positively associated with grain size. Thus, grain size is an important agricultural trait to improve the yield of rice. In addition, grain size affects not only yield but also quality [3]. Long and slender grains tend to be transparent and of edible quality, and thus highly prized in most rice-consuming regions globally. On the other hand, large grains show a relatively high ratio of chalky grains such as white belly or white core.

Over the past three decades, quantitative trait loci (QTL) mapping has provided a promising way to better understand the genetic regulation of yield traits in rice. More than 400 QTLs associated with rice grain shape traits have been identified, and nearly 30 genes have been cloned and demonstrated to control the traits of grain shape and size in numerous genetic studies. To date, several QTLs directly affecting grain size

have been cloned in rice, such as *GW2* [4], *GS3* [5], *qSW5/GW5* [6,7], *GS5* [8], *GS2* [9], *GL3.1/qGL3* [10,11], *GL7* [12], *GW8* [13], *TGW6* [14], *TGW2* [15], and etc. The identification and functional characterization of these genes have provided an important theoretical basis for the enrichment of genetic resources and the development of new breeding and cultivation strategies for rice.

An adequate molecular marker system is essential for molecular breeding and genetic analysis. Single nucleotide polymorphisms (SNPs) are a more stable, abundant, fast, and cost effective variation than other DNA markers. The Fluidigm SNP genotyping system has automated polymerase chain reaction (PCR) and integrated fluidic circuit (IFC) technology, which automatically mixes PCR reagents through microfluidic channel networks. The *indica-japonica* SNP assays, based on the Fluidigm system developed in the previous study [16], have been applied to various genetic analyses and molecular breeding, such as bulked segregant analysis (BSA) [17], genetic diversity analysis [18,19], QTL analysis [20–23], and background profiling [24–26]. This Fluidigm SNP marker set has provided a faster and more cost-effective tool than other high-throughput SNP genotyping systems for primary analysis during molecular breeding using inter-subspecific populations, to date.

In this study, the large grain line (LGL), selected in the breeding field due to its large grain size, was characterized based on morphological traits and the genome-wide *indica-japonica* SNP set. In addition, nine QTLs associated with grain size traits were identified in an F_2 population derived from a cross between LGL and Hanareum, using the *indica-japonica* SNP set. Furthermore, a novel QTL controlling grain thickness was validated in the selected $F_{2:3}$ population.

2. Materials and Methods

2.1. Plant Materials

An LGL showing enlarged grain length, width, and thickness was selected in the breeding field of a rice lab during line selection and was maintained in the rice lab of Pusan National University. Hyowon6 (a high-quality Korean *japonica* variety [27]) and Hanareum (a Korean high-yielding tongil-type variety [28]) were used for phenotypic comparison with LGL. To map the QTLs, the segregating populations were derived from a cross between LGL as a maternal parent and Hanareum as a paternal parent. A total of 94 F_2 and 186 F_3 individuals with parents were grown using conventional cultivation methods at the experimental farm of Pusan National University (Miryang, Korea) in 2018 and 2020, respectively.

2.2. Phenotype Evaluation

The phenotypic measurement of three traits related to grain size was performed after harvest. Harvested grain was air-dried and kept at room temperature before measuring grain size traits. 20 randomly selected, fully filled grains from each parent, $F_{2:3}$, and $F_{3:4}$, were used to evaluate grain length (GL), grain width (GW), and grain thickness (GT) at 0.01 mm precision, and the values were averaged as the measurements for each plant. The three traits were evaluated using vernier calipers. Statistical analysis was conducted using R software.

2.3. DNA Extraction

At the tillering stage, young leaf tissues from each plant were collected in the field. Total genomic DNA was extracted from leaves using the modified cetyltrimethylammonium bromide (CTAB) method as described by Murray and Thompson [29]. DNA concentration and purity were quantified and qualified using a spectrophotometer (NanoDrop One, Thermo Scientific). DNA samples with absorbance ratios of 260 nm and 280 nm (A260/A280) > 1.8 were diluted to a concentration of 50 ng/μL and used for Fluidigm genotyping.

2.4. Fluidigm Genotyping

A total of 192 *indica-japonica* Fluidigm markers for SNP genotypes [16], designed based on the genomic difference between two rice subspecies, were used for phylogenetic analysis of LGL and detecting QTLs associated with the three grain size related traits in the F_2 population derived from a cross between LGL and Hanareum. For validation of QTLs in the F_3 population, 11 *indica-japonica* Fluidigm SNP markers on target regions and 6 additional Fluidigm SNP markers developed in the previous studies [20,28] were used.

Fluidigm SNP genotyping was performed using the BioMark™ HD system (Fluidigm, San Francisco, CA, USA), 96.96 Dynamic Array IFCs for the F_2 population, and 192.24 Dynamic Array IFCs (Fluidigm) for the F_3 population, according to the manufacturer's instructions, at the National Instrumentation Center for Environmental Management (NICEM), Seoul National University (Pyeongchang, Korea). Fluidigm SNP Genotyping Analysis software was used to acquire genotyping results. All base calls were manually checked, and any errors in homozygous or heterozygous clusters were corrected before further analysis.

2.5. Phylogenetic Analysis

A total of 40 rice germplasms, including LGL, Hanareum, Hyowon6, and other germplasms genotyped using 190 *indica-japonica* SNP markers in a previous study [16], were used for phylogenetic analysis (Table S1). A total of 38 germplasms, not including LGL and Hyowon6, were selected and clearly classified into the specific subgroups of the previous study. A PowerMarker V3.25 [30] was used to calculate the genetic distance based on the CS chord [31] and to construct an unweighted pair group method with an arithmetic mean algorithm (UPGMA), which were visualized in Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7 [32]).

2.6. Linkage Map Construction and QTL Analysis

The QTL IciMapping 4.1 software [33] was used to construct the linkage map and QTL analyses. First, the BIN functionality (binning of redundant markers) was used to remove redundant markers. Markers showing more than 10% missing data were also removed. The output file obtained from the binning step was used for linkage map construction with the MAP functionality. The Kosambi mapping function was used to calculate genetic distances in centimorgans (cM) [34]. QTL mapping was carried out using the BIP functionality (QTL mapping in biparental populations). The inclusive composite interval mapping of additive (ICIM-ADD) QTL method with default option was used to detect additive QTLs. A significant logarithm of the odds (LOD) threshold value was calculated for each QTL using 1000 permutations at $p = 0.05$, and the LOD threshold for each QTL was ranged from 3.51 to 3.56.

3. Results

3.1. Characterization of LGL

LGL was selected and designated due to its large grain size. LGL showed a wider and longer grain size than Hyowon6 with a round type grain, and Hanareum with a slender type grain. In addition, the panicle and flag leaf size of LGL were also longer and thicker than the other two varieties (Figure 1).

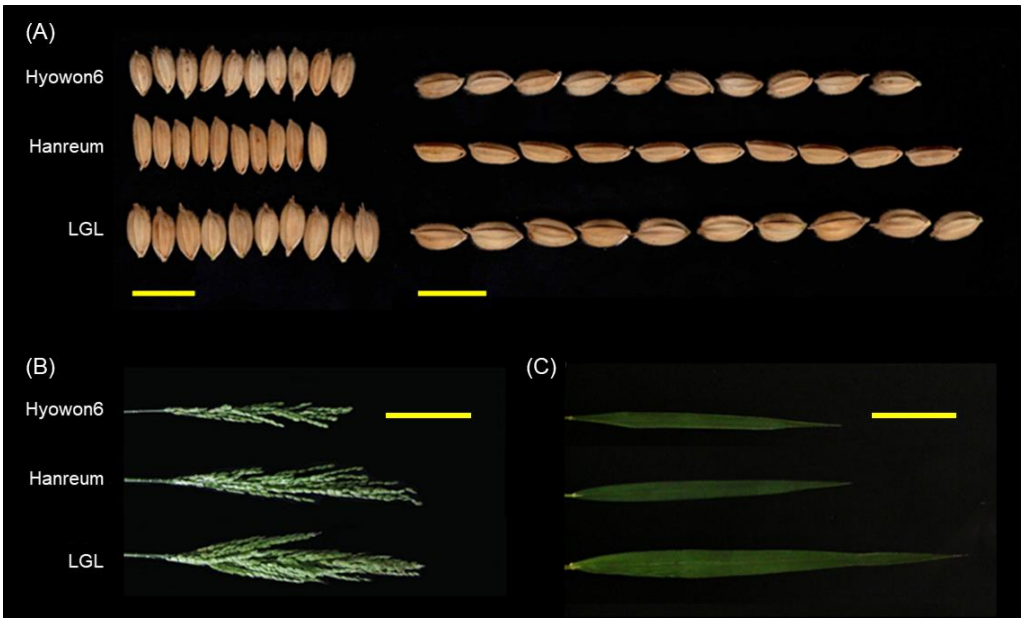


Figure 1. Morphological comparison of grain (**A**), panicle (**B**), and flag leaf (**C**) of Hyowon6, Hanareum, and LGL. Scale bar lengths are 10 mm, 10 cm, and 10 cm in (**A**–**C**), respectively.

We focused on the grain size of LGL and evaluated three grain size traits: GL, GW, and GT. The average of GLs for Hyowon6, Hanareum, and, LGL were 6.58 mm, 8.02 mm, and 8.15 mm, respectively. The GL of LGL was significantly longer than Hanareum, which has a long grain. For GW, Hyowon6, Hanareum, and LGM presented 3.20 mm, 2.95 mm, and 3.66 mm, respectively. The GW of LGL showed the largest value by a significant amount. The GTs of Hyowon6, Hanareum, and LGL were 2.12 mm, 2.00 mm, and 2.23 mm, respectively. Even for GT, LGL showed to be significantly thicker than the other two varieties (Figure 2). This result showed that LGL has a significantly large grain compared to normal varieties.

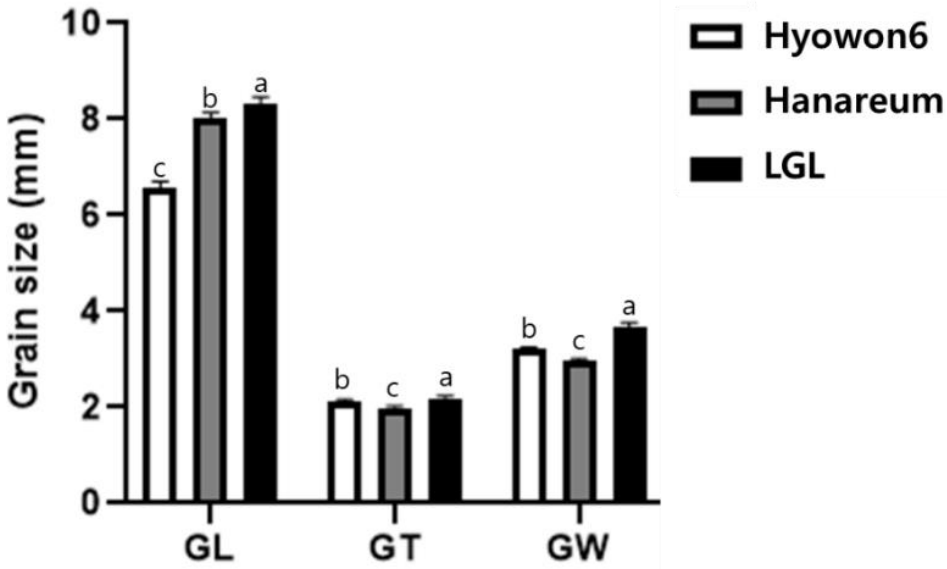


Figure 2. Phenotypic comparison of GL, GT, and GW. Duncan's least significant ranges (LSR) was used to identify significance. Significant differences at 5% level were presented by different letters such as a, b, and c.

LGL was an unstudied line with an unknown pedigree. To identify the genomic relationship of LGL with other varieties, phylogenetic analysis based on 190 *indica-japonica*

SNPs was conducted using a total of 40 germplasms including LGL, Hanareum, and Hyowon6. A total of 40 germplasms were divided into five subgroups, such as *indica*, *aus*, *aromatic*, tropical *japonica*, and temperate *japonica*, mirroring previous results [16]. LGL and Hyowon6 were clustered with temperate *japonica*, but LGL presented the largest genetic distance from Nipponbare, which is used as a reference variety for phylogenetic analysis, in the temperate *japonica* cluster (Figrue 3A).

LGL showed some *indica*-like introgression blocks (more than three continuous SNPs showing homozygous alleles different from Nipponbare) on chromosomes 1, 8, 9, and 12. This introgression pattern was unique to LGL (Figure 3B,C). This suggests that LGL is an untypical *japonica*-type line possessing a *japonica*-like genome with some *indica*-like introgression segments.

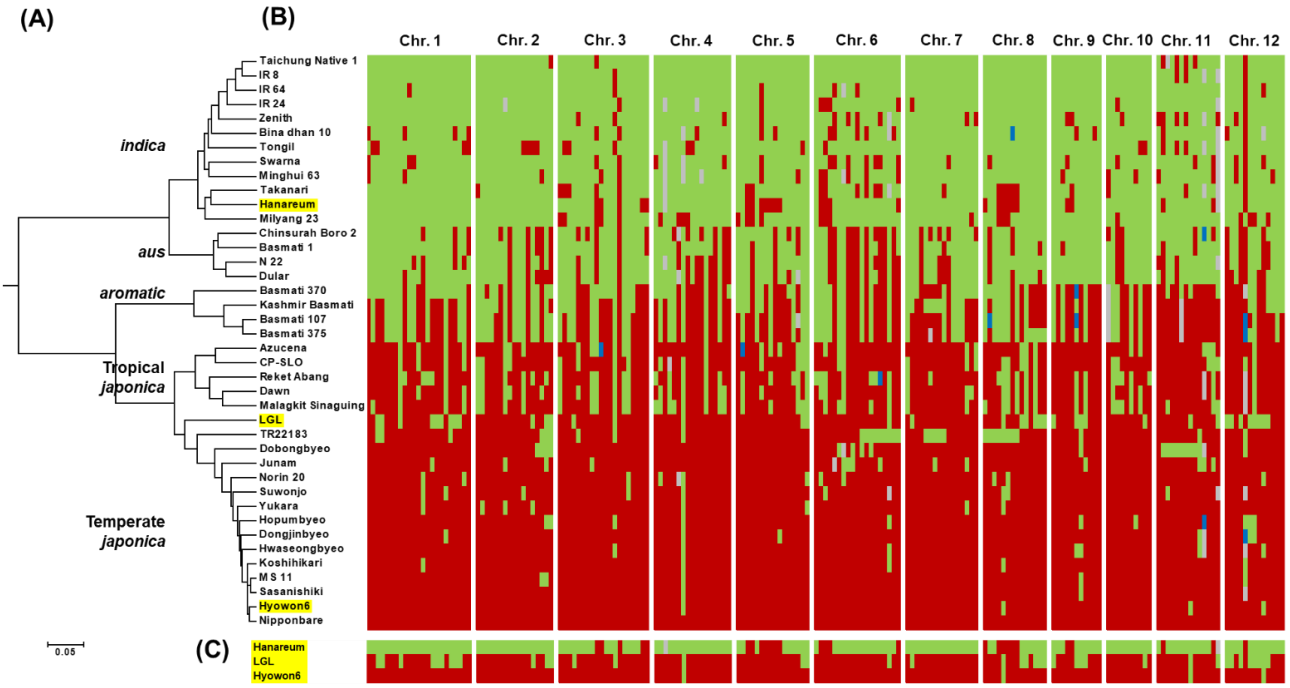


Figure 3. Phylogenetic analysis and whole-genome profiling of LGL using 190 *indica-japonica* SNPs: (**A**) UPGMA dendrogram of 40 germplasms; (**B**) allele distribution of 190 SNPs. Homozygous alleles identical to Nipponbare are shown in red and those different from Nipponbare are shown in green. Heterozygous alleles are shown in blue. Gray indicates missing genotype; (**C**) direct genotype comparison of Hanareum, LGL, and Hyowon6. Hanareum, LGL, and Hyowon6 were highlighted by yellow color.

3.2. Phenotypic Variation in the F_2 Population

GL, GW, and GT were evaluated in two parental lines and the F_2 population derived from a cross between LGL and Hanareum (Table 1 and Figure 4). All traits showed continuous distribution and a value less than 1.0 for skewness value, and this implies that all traits present approximately normal distribution. In addition, GL and GT showed bidirectional transgressive segregation in the F_2 population.

Table 1. Three traits related to the grain size of two parental lines and the F_2 population evaluated in the 2018 field.

Trait	LGL		Hanareum		F_2 Population					
	Average	SD	Average	SD	Average	SD	CV (%)	Range	Skewness	Kurtosis
GL (mm)	8.15	0.14	8.02	0.12	8.04	0.34	4.26	6.89–8.92	−0.25	1.01
GW (mm)	3.78	0.08	2.95	0.07	3.21	0.18	5.46	2.79–3.62	−0.04	−0.44
GT (ww)	2.23	0.05	2.00	0.04	2.11	0.1	4.75	1.85–2.37	0.1	0.24

Abbreviations are as follows: SD = standard deviation; CV = coefficient of variation.

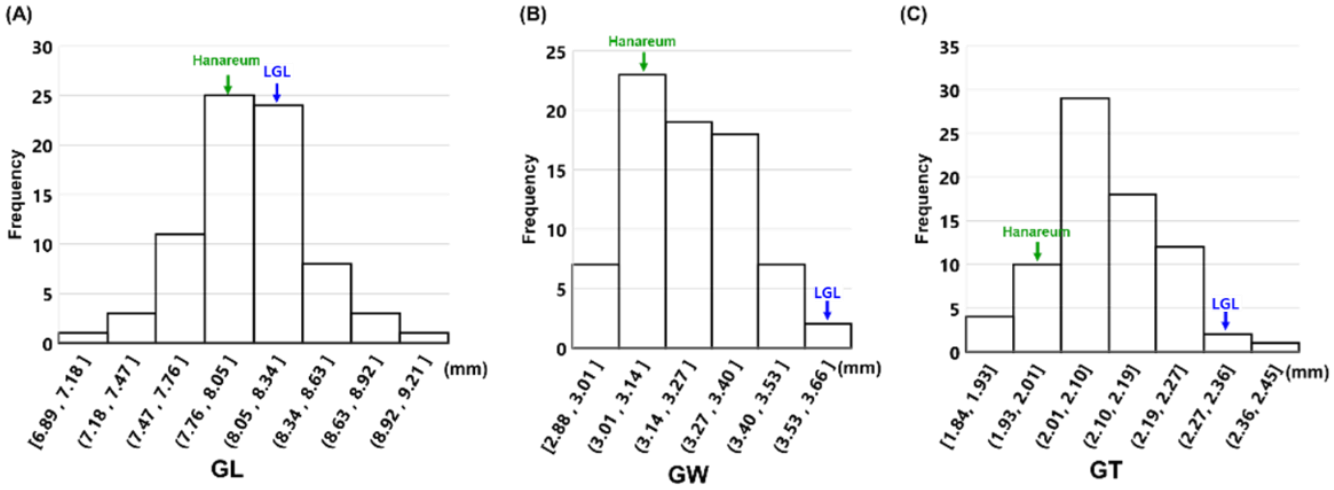


Figure 4. Frequency distribution for GL (**A**), GW (**B**), and GT (**C**) in the F_2 population derived from a cross between LGL and Hanareum.

LGL had a similar but significantly longer GL than Hanareum. The range and CV of GL for the F_2 population were 6.89 mm to 8.92 mm and 4.26%, respectively. For the GW, LGL was significantly wider than Hanareum, and the F_2 population had a range of 2.79 mm to 3.62 mm and a CV of 5.46%. Finally, GT was also significantly thicker in LGL than Hanareum and ranged from 1.85 mm to 2.37 mm in the F_2 population, with a CV of 4.75%.

Correlation analysis was carried out to identify the relationship between the three traits in the F_2 population. All pairs presented positive correlation. The highest and most significant positive correlation (0.586) was detected between GW and GT. The pair between GL and GT showed the lowest, and a nonsignificant, positive correlation (Table 2). This implies that the three traits, especially GW and GT, could be regulated by the same and/or tightly linked QTLs.

Table 2. Correlation coefficients among the GL, GW, and GT in the F_2 population.

	GL	GW
GW	0.208 *	
GT	0.162	0.586 **

* and ** indicate significant at $p = 0.05$ and $p = 0.01$ level, respectively.

3.3. Linkage Map Construction in the F_2 Population

The F_2 population, consisting of 94 individuals, was genotyped using 192 Fluidigm *indica-japonica* SNP markers. A linkage map of the F_2 population was constructed using 123 polymorphic and clearly genotyped SNP markers. The linkage map covered all 12 rice chromosomes with at least four markers for each chromosome. Several large genetic gaps showing larger than 30 cM intervals between adjacent markers were identified on eight chromosomes, not including chromosomes 2, 3, 4, and 10. The total length of the linkage map was 1318 cM and the average genetic distance between two adjacent markers was 10.7 cM (Figure 4).

3.4. QTL Analysis in the F_2 Population

To map the genomic regions controlling the large grain size of LGL, QTL analysis for three traits—GL, GW, and GT—was carried out using the phenotype and genotype data of the F_2 population derived from a cross between LGL and Hanareum. A total of nine QTLs were detected for the three traits on chromosomes two, three, five, six, and seven (Figure 5

and Table 3). Furthermore, we compared the genomic position of the QTLs detected in this study and previously reported QTLs/genes for grain size, based on the physical position of flanking markers for each QTL.

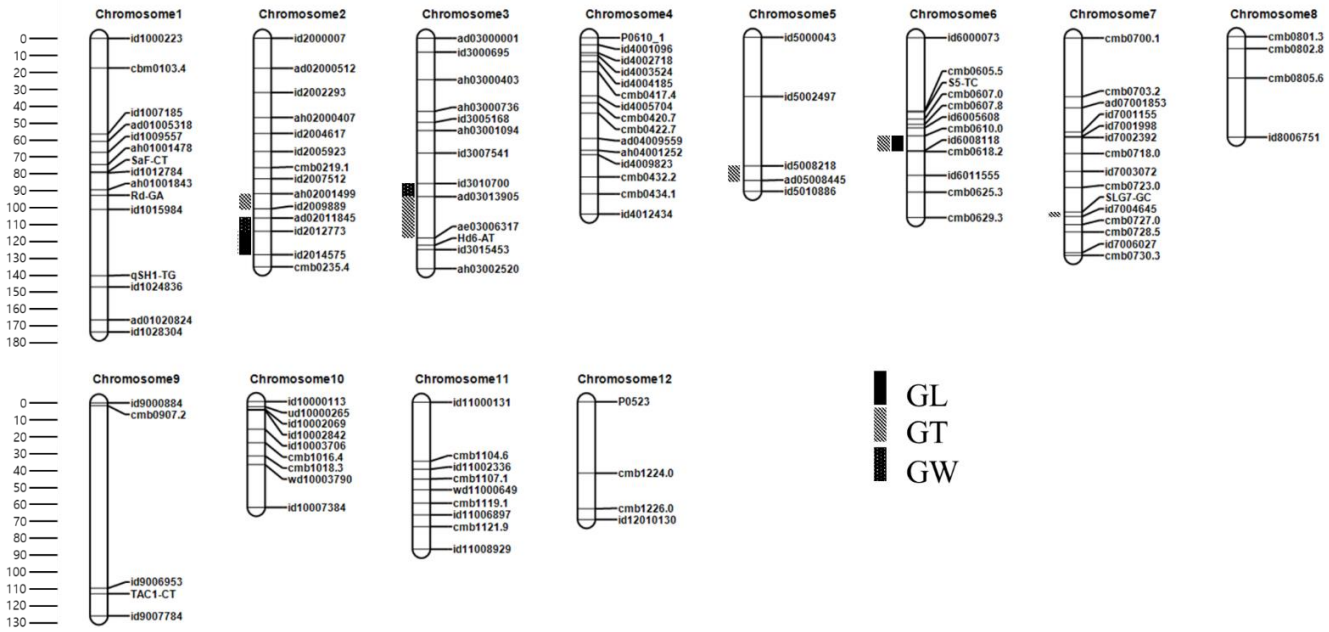


Figure 5. Linkage map and chromosomal locations of QTLs identified in the F_2 population. Chromosomes are numbered at the top and markers are listed on the right side of each chromosome. On the map, the leftmost sides indicate the scale of genetic distance (cM). Three types of rectangles located on the left side of chromosomes indicate the location marker interval of QTLs for three traits.

3.4.1. QTLs for GL

For GL, two QTLs were detected on chromosomes two and six, and these QTLs explained 17.26% and 16.15% of the phenotypic variation, respectively. These QTLs were responsible for 33.41% of the total phenotypic variation in GL. Alleles of LGL on *qGL2* and *qGL6* extended GL by as much as 0.2 mm and 0.28 mm, respectively. An atypical bHLH protein, which is involved in GL regulation and encoded by the *PGL2* gene, was reported in the genomic region of *qGL2* [35]. Furthermore, *qGL6* was overlapped with *gw-6*, a QTL for grain weight detected from a DH population derived from the inter-subspecific cross [36].

3.4.2. QTLs for GW

Two QTLs for GW were mapped on chromosomes two and three. *qGW2* and *qGW3* showed 24.27% and 23.53% of PVE, respectively, and LGL alleles on these two QTLs expanded GL by as much as 0.12 mm and 0.11 mm, respectively. *GS2* [9], a rare allele of this gene that enhances grain size including GW, was reported in the genomic region of *qGW2*. *qGW3* was overlapped with *gw3.1*, a QTL for grain weight detected in advanced backcross populations derived from crosses between *O. rufipogon* and *O. sativa*.

Table 3. QTL for grain size related traits detected in the F_2 population derived from a cross between LGL and Hanareum.

Trait	QTL	Chr.	Position (cM)	Left Marker	Right Marker	LOD	PVE (%)	Add	Reported Gene/QTL	Reference
GL	*qGL2*	2	130	id2012773	id2014575	4.5	17.26	0.2	*PGL2*	[35]
	qGL6	6	66	cmb0610.0	id6008118	4.2	16.15	0.28	*gw-6*	[36]
GW	*qGW2*	2	111	ad02011845	id2012773	6.22	24.27	0.12	*GS2*	[9]
	qGW3	3	94	id3010700	ad03013905	5.97	23.53	0.11	*gw3.1*	[37]
GT	*qGT2*	2	102	ah02001499	id2009889	5.88	13.24	0.05	-	This study
	qGT3	3	98	ad03013905	ae03006317	5.89	14.5	0.05	*GL3.1/qGL3*	[10,11]
	qGT5	5	79	id5008218	ad05008445	7.4	16.63	−0.06	N/A	[38]
	qGT6	6	60	cmb0610.0	id6008118	5.01	10.86	0.07	*gw-6*	[36]
	qGT7	7	108	id7004645	cmb0727.0	4.73	10.21	0.04	*SRS1/DEP2*	[39,40]

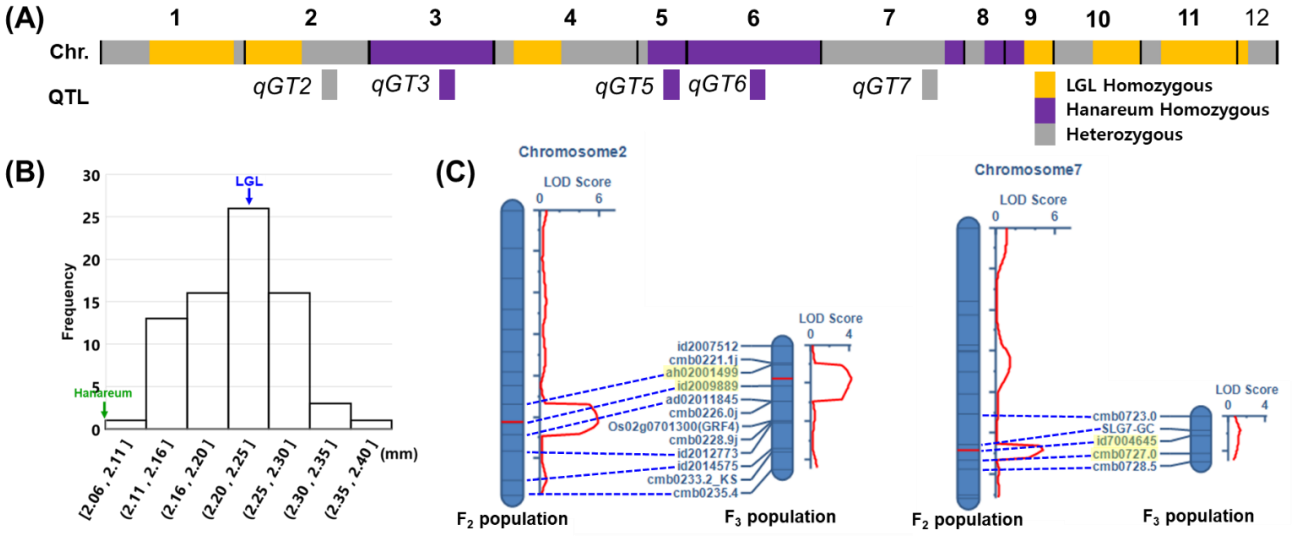
Abbreviations are as follows: LOD = logarithm of the odds; PVE = phenotypic variation explained by each QTL; Add = additive effect of LGL allele; N/A = not available.

3.4.3. QTLs for GT

A total of five QTLs for GT were identified on five different chromosomes. The PVE for each QTL ranged from 10.21% to 16.63%. Most of the LGL alleles on these QTLs increased GT. However, the LGL allele of *qGT5* showed a negative effect on GT. These QTLs explained a total of 55.43% of the phenotypic variation for GT in the F_2 population. The pleiotropic QTL affecting grain size traits, including GT, *GL3.1/qGL3*, was cloned to *OsPPKL1* in the genomic region of *qGT3* [10,11]. *qGT5* was overlapped with the QTLs for GT region, detected in the doubled haploid population from sake-brewing rice [38]. Furthermore, *qGT6* was located on the marker interval identical to *qGW6*, and overlapped with the previously reported grain weight QTL *gw-6* [36]. The grain size and shape regulating gene *SRS1/DEP2* was previously identified in the genomic region of *qGT7* [39,40]. There has been no QTL/gene related to GT found in the genomic region of *qGT2*, to date.

3.5. qGT2 Validation Using Selected F_3 Population

For the validation of *qGT2*, which is a novel QTL detected in this study, one F_2 individual possessing heterozygous alleles on *qGT2* and *qGT7* and Hanareum homozygous alleles on the other three QTLs was selected to develop the F_3 population (Figure 6A). The distribution of GT in the F_3 population consisting of 186 individuals showed normal distribution with a range of 1.97 mm to 2.36 mm. The variation of GT in the F_3 population was less than the F_2 populations (Figure 6B). A total of 17 Fluidigm SNP markers, including six additional markers on chromosome two, were used to construct linkage maps of the segregating target regions of the F_3 population (Figure 6C).



(D)

QTL	Chr.	Left Marker	Right Marker	F_2 population			F_3 population		
				LOD	PVE (%)	Add	LOD	PVE (%)	Add
qGT2	2	ah02001499	id2009889	5.88	13.23	0.05	4.14	10.52	0.032
qGT7	7	id7004645	cmb0727.0	4.73	10.21	0.04	-	-	-

Figure 6. Validation of *qGT2* using the F_3 population derived from a selected F_2 individual: (**A**) schematic genotype and alleles for five GT QTLs of a selected F_2 individual; (**B**) frequency distribution of GT in the F_3 population derived from selected F_2 individual; (**C**) linkage maps along with LOD graph of GT for chromosome 2 and 7. Blue dash line link same markers between linkage map of the F_2 and F_3 populations. Markers highlighted by yellow are flanking markers of *qGT2* and *qGT7*; (**D**) comparison of result of QTL analysis for GT in the F_2 and F_3 populations.

The QTL for GT in the F_3 population was detected on the marker interval identical to *qGT2* and explained 10.52% of the phenotypic variance. In addition, LOD graphs of the two segregating populations presented similar shapes, although several markers were added

to that region (Figure 6C,D). This suggests that the QTL detected in the F_3 population is identical to *qGT2* and stable to the environment and genetic background.

However, no QTL for GT was identified on the *qGT7* region in the F_3 population. This implies that *qGT7* could interact with other genomic regions and/or be affected by external conditions in order to express.

4. Discussion

Large grain size directly affects grain yield and quality, and is often considered a priority trait in specific rice breeding programs such as sake brewing [38,41]. LGL was selected according to its large grain size, but its panicle and leaf length phenotypes are also enlarged. Thus, LGL could be applied to develop whole crop silage rice. There are several reported QTLs/genes showing pleiotropic effects on grain size and plant architecture in the QTL regions identified in this study. For instance, *GS2* [9] and *SRS1/DEP2* [39], involved in not only grain size but also panicle architecture by regulating cell size and cell number, were located on *qGW2* and *qGW7*. LGL showed positive alleles on these QTLs, and it is considered that a large grain, panicle, and flag leaf could be contributed by these genes. However, we did not collect other phenotypes in the F_2 population and further genetic analysis accompanying detailed and repeated phenotype collection using an immortal population is required to elucidate this point. Thus, we have developed RILs derived from the F_2 population for the following study.

All the QTLs detected in this study showed the same direction, except *qGT5*, and at least two QTLs have clustered in similar positions on chromosomes two, three, and six (Figure 5, Table 3). This result could explain the correlation analysis that showed all positive correlation among the three traits. Especially, both QTLs for GW were located in positions less than 10 cM from the two QTLs for GT and GW, with GT showing the highest correlation coefficient by a significant amount (Table 2). This suggests that GW could be used as a selection trait for the large grain size of LGL.

Particularly, a novel QTL for GT, *qGT2*, was detected in this study. To validate this, one F_2 individual containing heterozygous *qGT2* was selected, and the F_3 population was derived from this individual. A QTL for GT in the F_3 population was identified on the same marker interval as *qGT2*. This result clearly indicated that this interval includes genes responsible for GT. A total of 360 genes were located in the genomic region of *qGT2* (Table S2). To clone the genes responsible for *qGT2*, the fine mapping of *qGT2* was performed using advanced segregating populations.

Genome-wide SNP marker sets have supported efficient phylogenetic analysis and polymorphic marker selection in this study. However, there were some genomic regions that markers did not cover due to a lack of polymorphism between LGL and Hanareum (Figures 3C and 4). This phenomenon was caused by the dissimilarity between LGL, Hanareum and the typical *japonica* and *indica*. A similar problem occurred using an RILs population derived from TR22183, a Chinese *japonica* possessing some *indica*-like introgressions (Figure 3B), and dasanbyeo, a Korean tongil-type variety containing some *japonica* introgressions [42]. To avoid this problem, identification of the genomic differences between parental lines based on whole-genome resequencing, and the development of additional markers, are required. Despite an incomplete linkage map of the F_2 population, the total PVE for each of the three grain size traits was more than 37% (Table 3). This implies that the Fluidigm *indica-japonica* SNP sets efficiently detected the QTLs for grain size in LGL.

Numerous QTLs related to grain size have been reported in rice, thus, identifying the novelty and validation of the detected QTLs is required before further study. In this study, we found an unstudied line showing large grain traits and carried out a QTL analysis of grain size traits. A relatively small F_2 population was used for the primary QTL analysis, and the selected F_3 population, which was segregated in the target QTL region, was used to validate the novel QTL detected in the primary analysis. This strategy could provide an efficient tool to profile the QTLs of target traits in unstudied germplasms.

5. Conclusions

The novel large grain line and QTLs related to grain size identified in this study could be applied to breeding programs developing large grain rice. A novel genetic locus for GT was detected and validated in this study, laying the foundation for further fine mapping and positional cloning to discover the mechanism of grain size regulation.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture11060565/s1, Table S1: Llist of 40 rice germplasms used for phylogenetic analysis; Table S2: List of genes located in the genomic region of *qGT2*.

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